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Conventional Cytogenetics and Fluorescence *In Situ* Hybridization in Persistent Cytopenias and Myelodysplastic Syndromes in Childhood

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Abstract. *Accurate detection of the abnormal clone in children with persistent cytopenia (PC) may confirm the diagnosis of myelodysplastic syndrome (MDS) and determine prognosis and evolution of the disease. Bone marrow (BM) samples were obtained from 65 children, 11 of which were finally diagnosed as primary or secondary MDS. Ten to 20 G-banded metaphases were analyzed and FISH was performed using a-satellite probes for chromosomes 7 and 8. Conventional cytogenetic analysis (CCA) was successful in 40/65 samples, revealing clonal aberrations in 3 patients with MDS. FISH was successful in all cases, detecting monosomy 7 and trisomy 8 abnormal clones in 5 patients. Abnormalities were identified in 3/6 children with primary MDS and 3/5 with secondary MDS. None of the patients with PC of etiology other than MDS had a clonal abnormality in the BM. The results confirm the high incidence of chromosome abnormalities in childhood MDS and the sensitivity of FISH in detecting minor abnormal clones.*

Persistent cytopenias (PC) represent a heterogeneous group of bone marrow (BM) failure disorders in childhood lasting for more than 6 months and are classified as inherited (congenital or not) and acquired (1). Despite the large number and diversity of the conditions that are associated etiologically, there is always a severe defect in the stem cells (SC) resulting in a disturbance of SC proliferation, division and differentiation rate, as well as reduction in the SC number (2). In the inherited conditions, patients have a variety of birth defects and a wide range in the hematological profile, from cytopenia involving one cell line (Blackfan-Diamond anemia) to aplastic anemia (Fanconi anemia). For most of the cases of acquired cytopenias (single or combined), a strong underlying mechanism of immune destruction of hematopoietic progenitors is noted, *e.g.* infections, autoimmune disease, drugs *etc.* (3).

Myelodysplastic syndromes (MDS) present with persistent cytopenia involving one or more lineages. They constitute a group of clonal SC disorders characterized by ineffective hematopoiesis and are primarily diseases of the elderly. In childhood they are rare, representing 4-5% of pediatric hematological malignancies. The annual incidence varies in population-based studies between 1.8-4 new cases /10^6 children under the age of 14 (4,5). Although the factors which play a major role in the pathogenesis of the disease remain unclear, it is almost certain that patients with BM failure syndromes such as Fanconi anemia and Shwachman-Diamond syndrome are at a higher risk (15,000-fold) for developing MDS (6-8).

The great heterogeneity of PC creates problems in diagnosis and classification of the disease. Clinical history and a thorough physical examination offer important information (9), but BM morphology and cytogenetic analysis are considered crucial steps for the diagnosis, prognosis and therapeutic approach (10,11). Detection of a clonal abnormality in the BM suggests a MDS (12).

In approximately 50-60% of children with primary MDS, non-random chromosome aberrations in the BM are detected with diagnostic and prognostic significance (10,11).

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Table I. *Reason for referral and underlying cause of cytopenia of the 65 patients included in the study.*

Reason for referral	No. of patients	Etiology of cytopenia	
Anemia	6	Primary MDS/RA	1
		s-MDS/RA	1
		Transient erythroblastopenia	2
		Viral infections (Parvo B19, HBV)	2
Neutropenia	9	Infections (Parvo B19, mycoplasma)	6
		Cyclic neutropenia	1
		Metabolic disease	1
		Immunodeficiency	1
Thrombocytopenia	16	Infections (EBV, CMV,brucella)	9
		Immune thrombocytopenic purpura	5
		Fanconi anemia	2
Anemia, thrombocytopenia	20	Primary MDS/RCMD	2
		Primary MDS/RAEB	2
		s-MDS/RAEB-t	1
		Infections (EBV,CMV,VZV virus, mycoplasma)	13
		Systemic Lupus Erythematosus	2
Anemia, neutropenia	13	s-MDS/RAEB	2
		s-MDS/RA 1	
		Infections (Parvo B19, influenza, mycoplasma)	8
		Juvenile Rheumatoid Arthritis	1
		Metabolic disease	1
Neutropenia, thrombocytopenia	1	Primary MDS/RCMD	1

Numerical aberrations are the most common, usually involving monosomy 7, trisomy 8, or trisomy 21 secondary to trisomy 8. Structural abnormalities include deletions, inversions or translocations, mainly of chromosomes 5 and 7. It is reported that chromosome 7 is usually involved in most abnormalities (30%), followed by chromosomes 8 (10%) and 5 (4,13).

Conventional cytogenetic analysis (CCA) of BM is the method of choice for the detection of an abnormal clone. Nevertheless, in many cases the culture is unsuccessful, because of the low rate of cell proliferation. Moreover, the poor quality and small number of metaphases spreads, due to pancytopenia, does not allow an accurate estimation of the neoplastic clone. FISH has had a large impact on the study of chromosome abnormalities in haematological malignancies. The ability to examine large numbers of non-dividing nuclei in a short period of time has made the detection of an abnormal clone more sensitive and accurate (14).

In the present study, the incidence of chromosomal abnormalities in children with PC of several etiologies, with emphasis on those with MDS, was evaluated by CCA and FISH techniques.

Materials and Methods

Materials. BM samples were collected and studied from 65 children (40 boys, 25 girls) aged 6 months to 18 years, followed at the Hematology-Oncology Unit of the 1st Department of Pediatrics, Athens University School of Medicine and the Department of Hematology-Oncology of "Aghia Sophia" Children's Hospital, Greece. Six of the 65 patients were diagnosed as primary MDS and 5 as secondary MDS (Table I). For the distinction between primary and secondary disease, any predisposing condition was taken into consideration (chemotherapy-radiotherapy for acute lymphoblastic leukemia, Hodgkin's, non-Hodgkin's lymphoma and congenital BM failure syndromes). The diagnosis and classification of MDS were made according to the WHO classification (15), based on morphological features of peripheral blood and BM, cytogenetic findings and the clinical history of the patients. The remaining 54 patients presented with PC of various etiologies involving one or two lineages (Table I). Viral infections, autoimmune or metabolic disease or Fanconi anemia were some of the underlying causes of cytopenia but, since the diagnosis of MDS was not clear, evidence of clonality by genetic studies was necessary.

Conventional cytogenetics. BM aspirates were cultured for 24 hours in RPMI-1640 culture medium (Invitrogen, Carlsbad, CA, USA) in a 5% CO_2 atmosphere at 37˚C and cells were exposed to colcemid solution before harvesting. Ten to twenty G-banded metaphases were analyzed from each patient and the karyotypes expressed according to the International System of Cytogenetic Nomenclature (ISCN 1995) (16). An abnormal clone was defined as: a) two or more cells with the same chromosome gain (trisomy), or the same structural abnormality, and b) three or more cells with the same chromosome loss (monosomy).

Fluorescence in situ hybridization. Dual color FISH was performed using directly-labelled commercial probes (Q Biogene, Strasbourg Cedex, France). The alpha-satellite D7Z1 probe for the centromere of chromosome 7 and the D8Z2 probe for the centromere of chromosome 8 were used according to the manufacturer's directions. Chromosome-specific hybridization signals were visualized through a "triple-band pass" filter (FITC/ rhodamine/DAPI) microscope. At least 200 nuclei were examined from each patient without any knowledge of the karyotypic data. Only isolated, non-damaged cells were counted and findings were interpreted according to the results of the pilot study.

Pilot study. Ten BM samples, obtained from normal donors (5 males, 5 females), were used to calculate the hybridization efficiency and normal cut-off values of the chromosome-specific probes. Donors were children that were being followed at "Aghia Sophia" Children's Hospital for minor hematological disorders, *i.e.* iron deficiency anemia. At least 300 nuclei were studied from each sample and the mean percentage of cells with normal (x_n) and abnormal (x_a) hybridization signals, standard deviations (S.D.) and cut-off values (C.V.) were calculated for each probe. Cut-off values were determined using the mean percentage of cells with abnormal hybridization signals plus three standard deviations (x_a +3S.D.) (17,18).

Table II. *Cytogenetic and FISH results in the bone marrow of MDS patients included in the study.*

No	WHO Classification	Age/sex	Primary disease	BM karyotype		FISH			
						D7Z1 (C.V. 15%)		D8Z2 (C.V. 10%)	
						2 signals	1 signal	2 signals	3 signals
1	RAEB	18m/F	None	48,XX+8+21 46,XX	90% 10%	93%	5 %	20%	65%
2	RCMD	4y/M	None	46,XYdel (11)(q21-qter) 46,XY	70% 30%	91%	5%	90%	6%
3	RAEB-t	18y/M	Hodgkin's lymphoma	46,XY, -7+8, t(5;17)(q35;q21) 46,XY	85% 15%	27% (-7) 18% (+8) 16% (-7+8) 39% of nuclei with normal signals			
4	RCMD	6m/F	None	-		77%	20%	89%	3%
5	RA	12y/M	ALL	-		96%	4%	88%	46 %
6	RAEB	8y/M	Shwachman -Diamond	-		92%	5%	82%	13%
7	RAEB	8y/M	None	46,XY		90%	7%	92%	4%
8	RA	7y/F	None	46,XX		93%	7%	92%	5%
9	RCMD	2y/M	None	-		95%	4%	96%	3%
10	RA	7y/F	ALL	-		93%	3%	95%	2 %
11	RAEB	9y/F	Fanconi anemia	46,XX		94%	4%	95%	3%

Results

Cytogenetic studies. CCA was successful in 40/65 BM samples (61.5%). The low rate of mitotic index and poor quality of metaphase spreads were some of the pitfalls of the method. In three patients (4.5%) clonal chromosomal abnormalities were identified, whereas in the remaining ones the karyotype was normal (Table II).

Patient No. 1 presented with anemia, thrombocytopenia, 5% blasts in peripheral blood and elevated HbF. BM showed erythroid and megakaryocytic dysplasia with 18% blasts. She was diagnosed as having refractory anemia with excess of blasts (RAEB) and had an abnormal clone 48,XX+8+21 in 90% of the cells analyzed. Patient No. 2, who presented with persistent anemia, thrombocytopenia and had 3% BM blasts with bilineage dysplasia, was diagnosed as having refractory cytopenia with multilineage dysplasia (RCMD). He had an abnormal clone [46,XY del (11)(q21-qter)] in 70% of the cells analyzed. The third child (No. 3) had received chemotherapy with alkylating agents for Hodgkin's lymphoma and developed secondary MDS (RAEB-t) five years later. He presented with anemia, thrombocytopenia, erythroid and megakaryocytic dysplasia in the BM with 25% blasts. The BM karyotype revealed three chromosomal abnormalities [monosomy 7, trisomy 8 and t (5; 17)(q35; q21)] in various combinations.

None of the patients with PC of etiology other than MDS had a clonal abnormality in the BM.

Fluorescence in situ hybridization. a) Pilot study: The mean percentage of cells with abnormal hybridization signals for probe D7ZI was 6%, the standard deviation of the sample 2.75 and the normal cut-off value was 15%. For probe D8Z2, the mean percentage of cells with abnormal hybridization signals was 3%, the standard deviation was 1.93 and the normal cut-off value 10%. Based on this

Table III. *Signal distribution after FISH analysis with D7Z1 and D8Z2 probes in the control samples.*

	0 Signal %	1 Signal %	2 Signals %	3 Signals %	Mean (%) Normal Distribution of Signals x_n	Mean (%) Abnormal Distribution of Signals x_a	S.D.	Cut-off Value % x_a +3S.D.
D7Z1	5 – 15	3 - 8	89 - 95	0 - 4	92.5	6	2.75	15
D8Z2	4 – 12	3 - 5	92 - 96	0 - 5	94.5	3	1.93	9

statistical analysis, an abnormal clone was defined as monosomic for chromosome 7 if more than 15% of nuclei examined exhibited one signal. A clone was defined as trisomic for chromosome 8 if more than 10% of the nuclei examined exhibited three signals (Table III).

b) Patients. FISH was successful in all cases and abnormal clones were detected in five patients (7.5%) (Table II).

In patient No. 4, who presented with persistent anemia, thrombocytopenia and met the criteria for RCMD, CCA was unsuccessful, due to the low mitotic index. FISH studies, in over 200 nuclei examined, revealed a clone with monosomy 7 in 20% of the cells.

Trisomy 8 clones were detected in two patients with secondary MDS, where CCA was unsuccessful. The first patient, (No. 5), presented with refractory anemia (RA) after chemotherapy for ALL and had an abnormal trisomic clone in 46% of total nuclei analyzed. The second (No. 6) was originally diagnosed with Shwachman-Diamond syndrome, a congenital BM failure syndrome, at high risk for myelodysplasia. At the time of karyotypic analysis, he had severe neutropenia, thrombocytopenia, and bilineage dysplasia with 10% BM blasts. He was diagnosed as having RAEB and an abnormal clone with trisomy 8 was detected by FISH in 13% of the examined nuclei.

Additionally, FISH analysis confirmed the cytogenetic findings in two patients (No. 1 and No. 3), although lower percentages of the neoplastic clones were detected (Table II).

In the remaining patients with PC caused by etiologies other than MDS, FISH testing for monosomy 7 and trisomy 8 was normal.

Discussion

PC with myelodysplastic features may occur in a variety of disorders of different etiologies, such as viral infections, mitochondrial cytopathies or autoimmune disease but, after treatment of the underlying disorder, the dysplastic changes generally resolve (19-22). The detection of a clonal cytogenetic abnormality in hematopoietic cells is helpful in confirming the diagnosis of MDS and plays an essential role in prognosis. It is, therefore, considered a crucial step for the selection of the most appropriate treatment plan (23-25).

In the present study, six of the sixty-five children (9%) that were studied were diagnosed as having primary MDS, whereas secondary MDS was confirmed in five patients (7.5%). The diagnosis of MDS was established according to morphological features, cytogenetic analysis of BM and peripheral blood findings, as proposed by the latest WHO classification of pediatric MDS (15, 26).

Among the six children with primary MDS, three (50%) had BM chromosome aberrations, mainly numerical. This is consistent with results from previous studies, which showed that non-random chromosome aberrations are seen in 50-60% of children with primary MDS (10,11,13). Aneuploidies were also noted in three of the five patients with secondary MDS (60%), although it is reported that the incidence of abnormal clones is higher in secondary MDS (> 85%) and hypodiploidy is more frequent (13,27). None of our patients developed a hypodiploid clone.

Monosomy 7 was detected as a sole abnormality, or part of a complex karyotype, in two patients. Trisomy 8 was observed in four and trisomy 21 in conjunction with trisomy 8 in one patient. Structural abnormalities, such as deletions and translocations, were detected in two patients (Table II). These findings are in agreement with previous reports, showing an increased frequency of aneuploidy for chromosomes 7 and 8 in pediatric MDS (10,11,12,27).

The fact that none of the patients with PC caused by reasons other than MDS had detectable chromosome aberrations in the BM strongly suggests the clonal nature of the disease.

Monosomy 7 and trisomy 8 were detected in a higher proportion in metaphases rather than in interphase cells. In two patients (No. 1 and No. 3) FISH detected lower percentages of the neoplastic clones. Possible explanations for these findings may be: a) the larger number of cells analyzed by FISH and b) the fact that CCA is restricted to actively dividing cells. It is known that neoplastic cells have a distinct proliferative advantage over the disomic normal cell population (28). Kibbelaar *et al.* also reported this tendency in about half of their cases (29). Furthermore, Wyandt *et al.* found that MDS patients exhibiting

monosomy 7 in 35-100% of metaphases examined showed a slightly lower range (9-90%) in FISH analysis (18).

The results obtained in the present study support the usefulness and advantages of FISH in conjunction with CCA for accurate study of the karyotype of the BM in pediatric MDS. CCA is necessary since it is highly informative and can detect not only structural, but also numerical abnormalities such as hypo-, or hyperploidy. On the other hand, FISH can reveal the presence of specific chromosome abnormalities when CCA fails, the neoplastic clone is small, or there is a submicroscopic deletion. It is well known that clones that cannot easily be detected may have a determinant role in the progression of the disease (30). For this reason, we intend to use additional probes from specific chromosomal regions for a thorough and more accurate evaluation of the neoplastic development of MDS.

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